UNIVERSITY OF PENNSYLVANIA

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A THESIS

IN BACTERIOLOGY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

PHILADELPHIA

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STUDIES ON THE WAXMOTH, GALLERIA MELLONELLA, WITH PARTICULAR REFERENCE TO THE DIGESTION OF WAX BY THE LARVAE

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TWO FIGURES

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INTRODUCTION

One of the micro-lepidoptera, the bee moth or waxmoth, Galleria mellonella, is a serious pest of bee hives. The larvae which hatch from the eggs of the moth burrow into seclusion in the comb where they are well protected from the bees. The larvae have a decided preference for the brood combs, where there is a great deal of nitrogenous material (Paddock, '29 a). They feed upon the comb and may reduce a hive to a mass of detritus and larval excreta in a very short time. Black bees seem to be less resistant to the attacks than Italian bees (Paddock, '29).

The distribution of the moth has followed the spread of beekeeping throughout the world (Paddock, '18). It is found throughout the United States, with the exception of high altitudes in the Rocky Mountains (Paddock, '26).

The loss to beekeepers from this pest is considerable. In 1911, in the state of Texas, 136 beekeepers reported losses varying from 5 per cent of their colonies to as high as 95 per cent (Paddock, '13).

BIOLOGICAL CONSIDERATIONS

The larva of the waxmoth or bee moth has been found very suitable for numerous investigations on immunity. The

blood is clear, containing no red cells, and the insect thrives at temperatures of 37° to 40°C. The temperature of the larva is normally higher than that of the surrounding air, and may exceed it by 17° to 22.7° (Uvarov, '31).

Chorine ('29) found the larva immune to tetanus toxin and capable of being immunized against diphtheria toxin. Métalnikov ('27), experimenting with over seventy species of bacteria, found the larvae very resistant to the most pathogenic organisms and particularly the tubercle bacillus.

It is extremely interesting that the organism which shows such a remarkable immunity toward the tubercle bacillus provided with a protective waxy capsule, is at the same time the only animal known to feed upon wax. Part of this investigation has been carried out to determine whether wax is actually utilized for food, and, if so, under what conditions this unusual digestion is brought about. It was also thought interesting to determine whether these hypothetical digestive principles were able to digest wax extracted from the tubercle bacillus.

EXPERIMENTAL

Determination of the digestion of beeswax by living larvae

Métalnikov ('08) refers to the wax separated from the nitrogenous material of honeycomb as consisting of two main constituents, with traces of a few insignificant substances. There is cerine, soluble in alcohol, and myricine which is insoluble in this solvent. The principal element of cerine is cerotic acid. Myricine is an ester of palmitic acid and myricyl alcohol. He states that this waxy material makes up 60 per cent of the honeycomb and only 28 per cent of the larval excreta, and concludes from these figures that the digestion of wax by the larvae accounts for the difference.

In this investigation experiments were carried out to determine the percentage of beeswax in comb, the percentage in pellets of larval excreta, and the percentage of pure wax that was lost in the passage through the larvae. In all experiments, brood comb received with the larvae from H. B. Parks,

of the Texas Agricultural Experiment Station, were used. Samples were selected for their uniformity of composition and structure. The determination of wax content of the specimens was carried out by means of ether extraction in the Soxhlet apparatus.

To determine the percentage of wax in comb, six specimens were used. The wax extracted from each sample was carefully weighed. The results were as follows:

Percentage of beeswax in uninfested comb

	Weight of comb Grams	*Weight of extracted wax* Grams	*Percentage of wax*
No. 1	2.000	0.763	38.15
No. 2	2.000	0.753	37.65
No. 3	2.000	0.821	41.05
No. 4	8.465	3.892	45.97
No. 5	6.897	3.070	44.51
No. 6	7.073	3.083	43.59

This gives an average percentage of wax in the comb of 41.82.

To determine the percentage of wax in excreta, pellets were very carefully removed from the detritus of a container in which waxmoth larvae were being reared, and in which practically all of the comb had been destroyed. The results were as follows:

Percentage of wax in larval excreta

Weight of pellets of excreta	*Weight of extracted wax*
2.218 grams	1.152 grams

These results show that the percentage of wax in the excreta is about 51.9, which is very close to the percentage of wax in the fresh comb.

Digestion of beeswax by waxmoth larvae

To determine whether beeswax is actually digested by the larvae, wax was removed from a quantity of comb in the Soxhlet apparatus and the remaining nitrogenous material, free of wax, was placed in flasks. Weighed quantities of commercial beeswax in thin shavings were placed over this

material. Larvae first starved for 24 hours were added, and the flasks placed in the incubator at 37°. The larvae were permitted to pupate in the flasks after complete ingestion of the wax. After carefully removing adults and pupae, the wax was extracted from the residue of the flasks, in the Soxhlet. Before all determinations the wax was dried in the oven at 100°. The results were as follows:

Weight of commercial beeswax added to wax-free comb, grams	*Weight of wax extracted from detritus and excreta, grams*	*Loss of wax, grams*	
5.865	2.871	2.994	51.0 per cent
14.607	7.516	7.091	48.5 per cent

No wax can be considered as lost in the pupae or adults, since the larvae empty their digestive tracts before pupating. The loss can only be explained by the theory that some of the wax was digested.

The wax seems to undergo no marked change in passing through the larvae, with the exception of reduction in quantity. Capillary tube melting point determinations were made of the wax before and after its passage through the larvae. Determinations gave a melting point of 62.5° for the former and 62.5° to 63.0° for the latter.

Métalnikov ('08), after a number of feeding experiments with waxmoth larvae, found that they could not exist on comb from which all wax had been removed. He found also that they were unable to increase in weight and size when fed upon pure wax. Since he also found that the larvae thrived on nitrogenous material which had been moistened with water, he concluded that the wax not only serves as food, but takes the place of water which is entirely lacking in the hive.

Experiments with wax of the tubercle bacillus

It was desired to determine whether wax from the tubercle bacillus is digested by the active principles in the digestive tract of the larva, and whether this wax can replace beeswax in the diet of the larva.

Buchanan and Fulmer ('28) summarize investigations up to that date on the composition of the waxy capsule of tubercle bacilli. They state that Ruppel, in 1898, found in the tubercle bacillus a wax showing some of the characteristics of beeswax. They find that, according to a number of investigators, the ether extract in per cent of dry weight in Mycobacterium tuberculosis, ranges from 26.2 to 40.8 per cent. Anderson ('27) separated the lipoidal constituents of human tubercle bacilli into three groups of substances, wax, glycerides, and phosphatides; the wax constituting about 50 per cent by weight of these extracts. Tamura ('13) isolated an alcohol to which he gave the name of mykol. He describes it as a monohydric alcohol with the formula $C_{29}H_{56}O$. The material is acid-fast and it, or one of its compounds, accounts for the acid-fastness of the organism.

Anderson and Chargaff ('29) isolated two new fractions consisting of two new fatty acids, tuberculostearic acid, $C_{18}H_{36}O_2$, and phthioic acid, $C_{26}H_{52}O_2$. The phthioic acid is biologically active (Anderson, '29). Sabin, Doan, and Forkner ('28) found this substance stimulated the formation of massive typical, but artificial tubercular tissue. The purified wax precipitated from ether and toluene by methyl alcohol gave similar reactions.

Digestion of wax of the tubercle bacillus by the larvae

A quantity of purified wax from human tubercle bacilli was obtained through the courtesy of the Mulford Biological Laboratories. It was the fraction A-6, the chloroform-soluble wax purified according to the method described by Anderson ('27). Some of this wax was placed in an oven at 110° for 24 hours. After cooling, the wax was found to weigh 1.122 grams.

To a solution of this wax in ether was added some comb from which all wax had first been removed by ether extraction. The flask was then placed in the oven until all the ether absorbed by the comb material was evaporated. Waxmoth larvae were added. The flask was covered with wire gauze

and placed in the incubator at 37°. The larvae seemed to thrive on this material, the greater part of which they ingested. They pupated and emerged as normal adults. The females deposited eggs which developed into normal young larvae. After 6 weeks, the remaining pupae were carefully removed. The material remaining in the flask was extracted in the Soxhlet and there was obtained 0.810 gram of wax. There was a loss of 0.312 gram of wax or 27.8 per cent.

Another quantity of the above wax weighing 1.014 grams was used in a similar experiment. Extraction of the material remaining in the flask after 5 weeks gave 0.899 gram of wax. This represented a loss of 0.115 gram, or 11.3 per cent.

A similar experiment was carried out with wax extracted in the laboratory from tubercle organisms received from The Henry Phipps Institute. About 150 agar tube cultures of human, avian, and bovine tubercle bacilli were obtained. The organisms were scraped off with a stiff wire loop. The weight of organisms (wet) obtained was 10.692 grams. The organisms were placed in the oven at 110° for 24 hours. The loss of weight at 110° was 8.290 grams, or 77.5 per cent. There remained 2.402 grams of dry cells.

The lipoids were extracted at room temperature with ether, and filtered through a Berkefeld filter. The dry cells gave 0.391 gram of ether extract. This total ether extract amounted to 16.3 per cent of the dry weight. To this was added ether as above and wax-free comb. The ether was then evaporated off in the oven. Larvae were added.

After 6 weeks, the remaining pupae were removed. Adults in all cases were permitted to escape upon their emergence from the cocoons. The extraction of the remaining material resulted in 0.363 gram of wax. There was a loss of 7.16 per cent of the original wax.

The above results would indicate that wax from the tubercle bacillus is utilized by the larvae. It is able to replace the beeswax of the normal diet satisfactorily. (In these experiments, also, larvae were starved for 24 hours before being added to the flasks, in order to permit them to excrete all wax

from their digestive tracts. Adults when removed were replaced with other larvae.)

The mechanism of wax digestion in waxmoth larvae

Although the digestion of wax by waxmoth larvae has been evident to a number of investigators, the means by which this digestion is brought about is not at all clear. Métalnikov ('08) found, in the intestine of Galleria, proteolytic enzymes, amylase acting on starch, lactase acting on milk, lipase acting on fats, but did not determine a specific enzyme acting on wax. He stated that it undoubtedly exists, saying that the intestinal extract acts upon wax.

Examination of the intestinal contents of the waxmoth larva discloses the wax in an unquestionably liquid condition. Numerous experiments were performed to demonstrate, if possible, action of larval or intestinal extracts upon beeswax, but all were unsuccessful. There was no action whatsoever by these extracts upon very thin films of wax under varied conditions of moisture and temperature.

An attempt was made to determine whether a larval extract had any effect upon the waxy capsule of tubercle bacilli, thereby altering its acid-fast properties.

Larvae obtained from Texas, weighing 9.6 grams, were ground up in a mortar with sterile quartz sand. Twenty cubic centimeters of sterile saline solution was added and the whole strained through a cloth. This was centrifugalized and the supernatant fatty and waxy material discarded. The sediment was also discarded. The material was placed in the refrigerator for 48 hours. Five cubic centimeters of this larval extract was then placed in each of two sterile tubes. Five-tenths per cent trikresol was added to one tube to inhibit the multiplication of a diplococcus which appears in practically a pure culture when the larval extract is incubated. Into each tube was added a generous loopful of avian tubercle bacilli. The tubes were placed in the incubator at 37° for 15 days. Material from the bottom of the tubes was examined for acid-fast organisms. The Ziehl-Neelsen stain was used.

Acid-fast organisms were numerous and they had apparently undergone no change.

The intestinal flora of waxmoth larvae

The digestion of unusual substances by certain insects has been explained in a number of interesting investigations as dependent upon the presence of certain intestinal microorganisms, existing under symbiotic conditions in the digestive tract. In such cases it has been shown that it is the enzymatic action of the microorganisms that brings about the digestion of the food material.

Cleveland ('24) has shown that digestion of cellulose by termites is brought about entirely by intestinal protozoa; the termites being unable to utilize this food material in the absence of these normal inhabitants of the digestive tract. Imms ('24) discusses an investigation by Portier, in which it was shown that in the case of the larva of Nonagria which lives within the stems of Typha devouring the pith, there are conidia of a fungus (Isaria) in the digestive tract living upon the devoured vegetable fragments. A micrococcus accompanying the conidia secretes an enzyme capable of digesting cellulose. Buchner ('28), finds the larvae of rose-chafers (Potosia cuprea) entirely dependent upon the presence of intestinal cellulose-digesting bacteria. Beckwith and Rose ('29) obtained cellulose digestion by bacteria from the digestive tracts of termites, but the investigations of Cleveland ('24) and Dickman ('31) indicate that their technique was inadequate.

In this investigation, a number of experiments were performed to determine, if possible, whether the intestinal bacteria which are numerous in the larvae are able to digest beeswax and thus account for the digestion of wax in the larvae.

Organisms from carefully sterilized larvae could not be isolated in tubes of inorganic nutrient solutions containing paper strips coated with beeswax, under aerobic or anaerobic conditions. (Examination of the digestive tract shows an un-

usual supply of large tracheal tubes, indicating an abundant oxygen supply, so that presumably any intestinal organisms are aerobic forms. All later experiments were carried out under aerobic conditions.)

Numerous pin point colonies of organisms were isolated, however, on agar plates, from the guts of larvae obtained from Pennsylvania. The organisms isolated were non-motile diplococci. They were shown to be numerous in hanging-drops from the larval gut, and to be present in apparently pure culture. They are spherical and about 0.6 μ in diameter. They appear singly and grouped in pairs. They are apparently destained by the Gram technique.

Agar colonies are circular, punctiform, with smooth, convex, and glistening surface. There was no apparent chromogenesis. On agar slants the growth was filiform, scant, and smooth. Stab inoculation gave a moderate filiform growth. There was a faint yellow growth on potato slants, which was the outstanding characteristic of this organism. With some strains which showed no growth on potato after 24 hours, repeated subcultures on potato revived this characteristic growth. There was no liquefaction of gelatin and no surface growth. Inoculation of litmus milk gave in 24 hours a decided acid reaction with reduction of litmus, and a soft curd in 48 hours. There was little or no growth in broth, no indol, no diastatic activity and no reduction of nitrates. Dextrose, lactose, sucrose, and generally mannite were oxidized with formation of acid and no gas. The optimum temperature was around 37°, making the organism adapted for growth in the larval intestine. The organisms were occasionally isolated from infested comb. The organism is not described in Bergey's Manual ('30).

In an attempt to encourage activity upon beeswax, a number of media were prepared to which were added materials normally not present in laboratory media, but which are present in the normal environment of the organisms and which might have some effect upon their metabolism. To tubes coated with thin films of beeswax were added a larval extract

medium made from ground larvae, or pollen extract medium made from ground aspen pollen, in nutrient salt solution or standard lactose medium (with indicator, pH 6.8). There was no effect upon the wax after more than 2 weeks, although microscopic examination of the media showed the inoculated organisms still present. No change in the wax was produced, using a modification of Fred & Peterson's yeast infusion peptone solution.

Examination of the intestinal contents of waxmoth larvae obtained in Pennsylvania showed a predominance of spherical organisms. Examination of the intestinal contents of larvae obtained from Texas showed a predominance of rod forms. All subsequent work was done with these rod forms, which were easily cultured in the laboratory, upon standard media.

Organisms from the gut of Texas waxmoth larvae

Wax coated tubes containing broth, inoculated with organisms isolated from pellets of excreta displayed rather noticeable changes after about 3 weeks. The wax was loosened from the tubes, and was either broken or broke up with very gentle shaking. The wax was distinctly translucent and not brittle. It was soft and folded very easily in the medium which was uniformly turbid. Control tubes which did not contain organisms did not show this general translucence. In the control the wax became rather white and opaque and remained so. When it was possible to break up the wax with violent shaking, the pieces were not soft, did not fold up like tissue, and appeared brittle.

Since, according to Métalnikov ('08) wax in the comb was the source of water for the larvae, it was believed that the liquid media used above would undoubtedly inhibit the action of the organisms upon the wax. In an attempt to encourage activity of the organisms upon wax, the following medium was used which is semiliquid at 37° and which at the same time contains no water.

Paraffin oil,	300 grams
Commercial beeswax,	100 grams
Casein (Pfanstiehl),	20 grams (Ash, 0.8 per cent)

The beeswax was first dehydrated in the oven. The whole was placed in the oven and baked for an hour. The weight of the medium was taken after sterilization. It was assumed that any organisms able to thrive in this medium would have obtained their water from the destruction of the beeswax, and this would have resulted in a corresponding loss of weight of the medium after drying in the oven. Material from the gut of larvae, or isolated organisms produced, after 3 weeks, no changes in flasks of the above media. No results were obtained in the above media to which pollen had also been added.

A paraffin oil medium was made, substituting peptone for the casein.

Paraffin oil,	300 grams
Beeswax,	100 grams
Peptone,	20 grams

The material was poured into Petri dishes and it remained firm at 37°, although the latter medium was practically identical with the former. Broth cultures of two rods isolated from the gut of Texas larvae, and designated later in this paper as T. 1 and T. 2, were added to the surface of the plates.

Four days later, the waxy substrate appeared opaque. There was evident penetration into the wax. There was a quantity of oil liberated on the surface. There was a distinct adherence of the broth to the surface of the wax. The surface of the wax was dulled and roughened. The control plate in which there was sterile broth was only slightly opaque. The surface of the wax was still smooth, and a small quantity of oil was liberated to the surface of the broth. Nine days later, when the plates were washed with cold water, the control showed no decided etching. Plates inoculated with T. 2, and some with T. 1, showed a decided etching and marked irregularities of surface. Penetration into the wax was very decided in all plates, with these changes due entirely to the presence of the microorganisms.

Organisms found to be abundant in Texas larvae, and isolated

Four distinct rods, designated as T. 1, T. 2, T. 12, and T. 13, were isolated and used in the following experiments. T. 1, was found to be abundant. T. 12, was an organism difficult to cultivate and little work was done with it. Organism T. 13 corresponded very closely with the diplococcus isolated from larvae of Pennsylvania waxmoths. Systematic studies were made of these organisms after incubation at 37.5°.

Organism T. 1, was an actively motile, Gram positive, spore-forming rod measuring about $1 \times 2.4\ \mu$. In standard broth there was moderate to abundant turbidity, with a pellicle which settled to the botton. On agar slants there was abundant, smooth, moist, creamy, raised, glistening, surface growth, with the stab inoculation giving a very faint filiform growth. Litmus milk was peptonized, with no acid or curd after 4 days. There was crateriform to complete liquefaction in gelatin after 3 days. The potato slant displayed an abundant, moist, creamy growth with a raised lobate margin. Growth after 4 days was creamy yellow. Nitrate was reduced to nitrite. Starch was hydrolyzed. Indol was formed after 4 days. Acid was produced in sucrose, dextrose, and maltose, but not in lactose and mannite.

Organism T. 2 was a short rod $1 \times 0.5\ \mu$. It is non-spore-forming and partially destained by Gram's. It does not appear to be motile, the organisms tending to clump. In broth a thin entire pellicle was formed which still floated after agitation. There was no turbidity and no sediment. The agar slant showed no stab growth, with the surface growth entire, flat, dull, and smooth. Litmus milk was peptonized in 6 days; no acid, but discolored by a light plum-colored pigment. There was slight crateriform liquefaction in gelatin after 3 days. A potato slant was distinctly grayed in color by a pigment, with the moderate moist growth and potato becoming olive-colored in 4 days. Nitrate was not reduced. Starch was not hydrolyzed. No indol was formed. Acid was produced in sucrose and dextrose, very slightly in mannite, and none in lactose and maltose.

Organism T. 12 was a short motile rod, $0.5 \times 1\,\mu$. It was Gram-positive. A thin entire pellicle was formed in broth. The pellicle contained small granular masses and floated upon agitation. The agar slant showed an abundant flat growth, with a lobate margin dull at the periphery. Litmus milk was completely reduced. There was a complete thick curd after 4 days, with the upper seventh of the tube giving an acid reaction. There was a slight crateriform liquefaction of gelatin after 3 days. Growth on potato was abundant, raised, rugose, dry, cream-colored, with the growth and potato lightly browned after 4 days. Nitrate was reduced. Starch was hydrolyzed. Indol was not formed. Acid was produced in sucrose, lactose, dextrose, maltose, and mannite.

Relation between intestinal organisms and wax digestion

Evidence has been advanced by a number of investigators that living active bacteria cause the splitting of fats. It has been shown that a number of organisms, such as B. pyocyaneus, Staphylococcus aureus, B. fluorescens, and others, split beef tallow into fatty acids and glycerin in plate cultures.

Wells and Corper ('12) carried out a number of experiments to demonstrate that bacteria contain intra-cellular enzymes showing definite lipolytic activity. They found bacterial lipases similar to the lipases of mammalian tissues in being inhibited by sodium fluoride in high dilutions. They conclude that the similarity of behavior is so great that there is an indication of the similarity or identity of the lipases of bacterial and mammalian tissues.

Their procedure consisted in taking large quantities of bacteria to be tested from cultures on agar in large flat bottles. They used from 40 to 80 such cultures of each organism. After maximum growth had been reached, they scraped the organisms off into toluene water, or washed them off with toluene water. The suspension was kept at room temperature for 24 hours or longer, at which time subculturing indicated that all organisms were killed. This also induced a certain amount of autolysis and the liberation of endocellular

enzymes. Samples of the emulsions were taken and the dry weight of the contained bacteria determined. The emulsions were then divided into two equal portions and one heated for ½ hour submerged in boiling water. The latter served as the control. To each pair of flasks was then added a portion of the ester to be tested, olive oil, ethyl butyrate, or triacetin, a slight excess of toluene, and a few drops of phenolphthalein solution. The mixture was made neutral with N/10 NaOH, tightly stoppered and put in the incubator. At intervals the mixture was neutralized again with N/10 NaOH, and it was determined in this manner to what degree the esters had been hydrolyzed by the various organisms. Considerable hydrolysis was observed.

Working with B. pyocyaneus, Staphylococcus aureus, B. coli communis, B. dysenteriae (Flexner), and B. tuberculosis, these observers found that each bacterium exerted a corresponding effect upon each of the esters used, and suggested that the same enzyme is concerned in each case. They found also that there was some activity displayed by cultures heated for 30 minutes at 100° and interpret this as due to the presence of a thermostabile lipase.

Hydrolysis of beeswax by organisms from Texas waxmoth larvae

A modification of the above procedure was first used to determine whether intra-cellular enzymes capable of hydrolyzing beeswax were present in organisms isolated from the digestive tracts of waxmoth larvae. In these experiments small quantities of organisms were prepared according to the method of Wells and Corper, and placed in rolled-wax test tubes. Hydrolysis was determined by change in pH in the tubes.

Organisms were raised on standard agar plates or upon yeast extract agar. The yeast extract agar was made in the following manner:

Fleishman's yeast,	10 grams
Peptone,	10 grams
Na_2HPO_4,	5 grams
Water,	500 cc.

This was heated in flowing steam for ½ hour, then adjusted to pH 7.6. (The pH of the standard agar medium was 6.8.) This was then boiled for 1 minute over an open flame and filtered through a porcelain filter and filter paper under pressure, after diatomaceous earth had first been added to clarify it. To the filtrate was added 500 cc. of a 3 per cent agar solution. Twenty-four hour cultures of the organisms were used.

Aqueous suspensions were made with distilled water. One-tenth of a cubic centimeter of toluene was added to each 5 cc. of suspension. The suspension was agitated and 10 cc. placed in a rolled wax tube. Ten cubic centimeters was placed in a plain tube and kept as a control. All were incubated at 37°. pH readings were made with the quinhydrone electrode. Twenty-four hour readings were made. They were:

Organism, source		*pH, after 24 hours*	*Loss in pH*
T 1	Standard agar	6.01	
	Control	7.45	1.44
T 1	Yeast extract	7.37	
	Control	7.70	0.33
T 1	Standard agar	7.53	
	Control	8.04	0.51
T 1	Yeast extract	7.51	
	Control	7.79	0.28
T 1	Standard agar	7.55	
	Control	8.36	0.81
T 1	Standard agar	7.72	
	Control	8.04	0.32
T 1	Standard agar	7.70	
	Control	8.26	0.56
T 2	Standard agar	7.87	
	Control	8.21	0.34
T 2	Yeast extract	7.27	
	Control	7.87	0.50
T 2	Standard agar	7.65	
	Control	8.14	0.49
T 12	Yeast extract	7.45	
	Control	7.87	0.42
T 12	Standard agar	7.55	
	Control	7.80	0.25
T. 13	Yeast extract	7.06	
	Control	7.37	0.31

The loss represents the difference in pH between the wax tube and the control. It was assumed that this loss was due to the activity on beeswax of substances released from the bacterial cells. Using another type of control, a rolled wax tube and distilled water, showed a difference of only 0.13 in pH after 24 hours' incubation.

To determine whether any change could be produced in pH by living organisms; i.e., whether any extracellular enzymes were produced, the following experiments were performed. The following medium was made up:

H_2O,	1000 cc.
NaCl,	5 grams
$MgSO_4$,	1 gram
K_2HPO_4,	1 gram
Peptone,	10 grams

This was adjusted to pH 7.73 by the quinhydrone electrode. The medium was placed in rolled wax tubes, and inoculated with organisms. The tubes were kept at 37°. The control tube contained no organisms.

Readings after 24 hours:

Organisms	*pH*	*Loss in pH*
Control	7.73	None
T 1	7.73	None
T 1	7.65	0.08
T 1	7.65	0.08
T 1	7.79	None
T 1	7.70	0.03
T 1	7.70	0.03
T 1	7.87	Gain
T 2	7.40	0.33
T 2	7.40	0.33
T 12	7.34	0.39
T 12	7.67	0.06
T 13	7.89	Gain
T 13	7.94	Gain
T 13	7.97	Gain
T 13	7.80	Gain

Organism T 2 and, to some extent, T 12 were the only ones showing any appreciable lowering of pH, comparable to that caused by the endocellular substances of the organisms in general. With this medium acidity could only have been

produced to any considerable extent by the breaking up of the wax, so that T 1 and T 13 obviously did not liberate extracellular enzymes in this medium.

A medium was made up as above, but in which the peptone was replaced with 1 gram of urea. The organisms grew in this medium, and final pH readings showed a more uniform loss. The final pH was compared with that of the control.

Organisms	*pH*	*Loss in pH*
Control	8.72	None
T 1	8.43	0.29
T 1	8.47	0.25
T 1	8.47	0.25
T 1	8.43	0.29
T 2	8.57	0.15
T 12	8.47	0.25
T 13	8.82	Gain
T 13	8.79	Gain

All of the experiments up to this time were carried out with small amounts of media and with very small numbers of organisms. A series of experiments were carried out following more closely the procedure of Wells and Corper, in which large quantities of organisms were used.

Liberation of acid in flasks containing beeswax and organisms

In the following experiments, organisms T 1 and T 2 were used, because it was not difficult to obtain an abundant growth which was at the same time easy to wash off from the plates. Twenty-four hour cultures from standard agar plates were used. The number of plates averaged about twenty-four for each test. The organisms were washed off into a flask with distilled water. A portion of this was dried in the oven at 110° to determine the dry weight of the organisms used in each experiment. To each suspension was added enough water to make a volume of 100 to 150 cc. Enough toluene was added to saturate the water and provide a little excess. The whole was agitated and set aside at room temperature for 24 hours. A few drops of phenolphthalein solution was added and enough dilute NaOH for neutralization. The suspension

was now divided into two equal quantities and placed in flasks, each of which contained 5 grams of thin flakes of commercial beeswax. One flask was heated in a water bath for 30 minutes to destroy, if possible, thermolabile enzymes. From time to time any acid formed in the tubes was neutralized with N/10 NaOH. Whenever necessary, additional small quantities of toluene were added to the controls after heating to make up for the loss.

The following table indicates the total amounts of N/10 NaOH, in cubic centimeters, added up to each date to keep the acid liberated neutralized.

From the table it can be seen that there was a progressive production of acid in the flasks. Although there was at the same time acid liberated in the controls, the quantity at all times was less than that in the unheated flasks. It might be concluded that there were substances in the cells not inactivated by heating.

The liberation of acid in the unheated flasks was not quite so striking as the change brought about in the appearance and composition of the wax. The wax in the presence of bacterial material, and even to a great extent in the presence of material which had been autoclaved as a control, was greatly disintegrated. This was never the case in flasks in which the bacterial material had been omitted.

Other changes brought about in beeswax in presence of organisms

Five grams of commercial beeswax was placed in a 100 cc. Erlenmeyer flask together with distilled water (about 60 cc.). Toluene and four drops of phenolphthalein were added. The material was made neutral with N/10 NaOH. In 31 days only 1.1 cc. of the above NaOH was necessary to neutralize the material from time to time.

To another flask containing 5 grams of beeswax, toluene water, and phenolphthalein, was added the equivalent of 0.259 gram dry weight of organism T 1. After 24 hours, the material was neutralized with NaOH. The acid formed was neutralized from time to time for 5 days.

Total amounts of N/10 NaOH in cubic centimeters added up to each date

ORGANISM	VOLUME OF SUSPENSION	DRY WEIGHT	1ST DAY	2ND DAY	3RD DAY	4TH DAY	10TH DAY	11TH DAY	21ST DAY	TOTAL CC.	DIFFERENCE BETWEEN CONTROL AND TEST
	Cc.	*Grams*									*Cc.*
T 1	75	1.071	1.25	3.75	8.13	10.75	18.51			18.51	
Control T 1	75	1.071	0.00	0.25	2.75	6.63	11.25			11.25	7.26
T 1	50	0.360		6.13	10.01	12.64	18.27		24.27	24.27	
Control T 1	50	0.360		2.50	4.38	5.88	10.26		15.52	15.52	8.75
T 2	75	0.665		4.63	7.63	9.13		16.13		16.13	
Control T 2	75	0.665		1.50	3.00	4.13		6.76		6.76	9.37
T 2	50	0.333		4.63	8.10	9.60	12.48		19.36	19.36	
Control T 2	50	0.333		2.13	4.01	5.26	9.26		14.14	14.14	5.22
T 2	50	0.252	3.63		4.88	6.13	9.51		11.26	11.26	
Control T 2	50	0.252	1.75		2.38	3.01	5.52		7.40	7.40	3.86

The following photographs show very clearly the nature of the materials in the two flasks; one shows the wax from the flask containing no organisms, after 31 days, and the wax from the flask containing the organisms, after only 5 days; the other shows the wax spread out on a glass slide 1×3

Fig. 1 Left flask, containing wax and organisms, after 5 days. Right flask, containing no organisms, after 31 days.

Fig. 2 Upper slide, waxy material from above flask containing organisms. Lower slide, particles of wax from control flask containing no organisms.

inches. The wax from the flask containing bacterial material can be seen entirely disintegrated, and very much softened and broken up. The flakes were also much bleached in appearance. On the other hand, the wax flakes from the flask containing no organisms has undergone no change in appearance.

In the photograph of the flasks, the unchanged flakes of wax can very easily be recognized in the flask containing no organisms, while in the other the minute particles of material can be seen on the walls. Both flasks were kept for the entire period of time at 37°.

DISCUSSION OF RESULTS

Numerous investigators have proclaimed the remarkable immunity of the waxmoth larvae toward tubercle bacilli, which has been explained in part by the lipolytic activity of the blood and leucocytes.

Quantitative experiments discussed in this paper indicate that this is accompanied by unquestionable digestion of beeswax in the digestive tracts of the larvae. This digestion has not up to the present time been proved due to the production of enzymes by the larva itself. The wax, nevertheless, in its passage through the digestive tract is emulsified and assimilated; approximately 50 per cent of the wax being consumed in this manner.

Waxy material obtained from the tubercle bacillus has been shown to be an adequate substitute for beeswax in the diet of the larvae. This material, which has been shown to be biologically active for the usual laboratory animals, has no detrimental effect upon the larvae and they seem to thrive upon this substitute. It is, no doubt, true that the active materials which digest the beeswax act upon this material also, and it would seem to be true that the wax of the tubercle bacillus is utilized in the normal metabolism of the larvae.

The presence of active principles in the bodies of waxmoth larvae produced probably by the intestinal organisms, in whole or in part, capable of digesting the waxy material of tubercle bacilli make the larvae of unusual interest, since it is undoubtedly due to the absence of such principles in human beings and higher animals that the latter fall prey to this parasite.

In recent investigations with the use of the live maggots of flies in the treatment of chronic osteomyelitis, it has been

observed that these larvae, besides mechanically clearing away diseased tissue and making the wound alkaline and inhibiting the growth of pathogenic organisms, at the same time elaborate substances inimical to bacterial growth. Livingston and Prince ('32) obtained this substance or 'active principle' from fly maggots by grinding them up in saline and filtering the material. In a series of remarkable experiments these investigators find that the use of this material in the treatment of chronic osteomyelitis, infected wounds, and compound fractures gave astounding results.

It would seem very interesting to determine whether extracts of waxmoth larvae, intestinal organisms, or both, contain the principle which is vital in the destruction of the waxy capsule of the tubercle bacillus, and whether inoculations of this material have any effect upon the resistance of laboratory animals toward the tubercle bacillus. Experiments along this line are being undertaken at the present time.

Microorganisms were found abundant in the digestive tracts of the waxmoth larvae. Some of these which appear to thrive in crushed larvae could not be grown in the laboratory in sufficient quantities on the usual laboratory media for the experiments carried out.

The organisms isolated seemed to produce substances, apparently esterases, capable of breaking up beeswax to some extent, with the liberation of acid. The most striking effect, however, was the effect upon the structure of the wax. It would seem very possible that even though the activity of intestinal organisms may not account for the complete breakdown of the wax, they may be responsible for the production of intermediate substances which may then be digested and assimilated by the larvae.

SUMMARY AND CONCLUSIONS

It has been determined that bee comb, upon which waxmoth larvae have been found to thrive, contains, on the average, 41.82 per cent of beeswax.

Excreta of waxmoth larvae has been found to contain about 51.9 per cent of beeswax. All of the wax ingested by the larvae is not assimilated, the percentage of the wax in the excreta being very close to that in the ingested comb.

Of the wax passing through the digestive tracts of larvae, about 50 per cent is emulsified, digested, and apparently assimilated. No change was detected in the wax excreted by the larvae.

Purified wax obtained from tubercle bacilli is a suitable substitute for beeswax in the diet of the larvae. It has no toxic effects upon the larvae and is apparently assimilated.

Larval extract had no effect whatsoever upon beeswax. This extract had no effect upon the acid-fastness of tubercle bacilli.

Numerous bacteria were found inhabiting the digestive tracts of waxmoth larvae. Experiments indicate that active substances probably esterases, are produced by these microorganisms which cause marked changes in beeswax in vitro, and which might play an intermediate part at least in the digestion of wax by the larvae.

The ability to digest beeswax and wax from tubercle bacilli may account for the resistance of the larvae to infection by this organism and intoxication by its active components.

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